Lack of correlation between phenotype and genotype in untreated 21-hydroxylase-deficient Indonesian patients

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Clinical Endocrinology (2009) **71**, 628–635 doi: 10.1111/j.1365-2265.2009.03550.x

ORIGINAL ARTICLE

Lack of correlation between phenotype and genotype in untreated 21-hydroxylase-deficient Indonesian patients

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Summary

Background Mutations in *CYP21A2* lead to deficiency of 21-hydroxylase and can have either severe or moderate effects on phenotype, which can be prevented by early treatment. We studied long-term effects of this deficiency on phenotype in patients who had not been treated for prolonged periods and correlated these phenotypes with the mutations found in our patients.

Objective To assess the correlation between genotype and phenotype in untreated patients with 21-hydroxylase deficiency.

Design Subjects with 21-hydroxylase deficiency were selected from a large population of Indonesian patients with disorders of sexual differentiation. *CYP21A2* mutations in these patients were correlated with their phenotype in terms of genital development and steroid hormone levels.

Patients Fifteen 46,XX patients with ages between 1 and 33 years, of whom 12 had never been treated before.

Measurements Mutations in *CYP21A2*, genital phenotype and steroid hormone levels.

Results We found in all patients *CYP21A2* mutations which affect enzyme activity, with a relatively high allele frequency of R356W (40%), I172N (20%) and IVS2 - 1A > G (13%). Clitoris length was directly correlated with levels of testosterone, but not with age. The phenotype was not always concordant with the genotype: different phenotypes (mild to severe virilization) were found in sibling pairs with the mutations IVS2 - 13A > G or I172N. The high frequency of homozygous mutants for R356W in patients aged from 1 to 11 years old is remarkable, as this mutation has been described only in salt-wasting patients. In our study, this mutation caused a urogenital sinus in three out of seven cases, whereas in the remaining cases the labia were at least partially fused. This mutation caused severe virilization with remarkably high serum levels of renin. We found one novel substitution in intron 2 (IVS2 - 37A > G), containing the branch site, which is likely to affect the CYP21-enzyme. Two additional intron 2 substitutions were discovered, which are supposed to affect the 21-hydroxylase (i.e. IVS2 + 33A > C and IVS2 + 67C > T).

Conclusion We conclude that a correlation exists between the concentration of androgens and the extent of virilization. However, there was no clear correlation between genotype and phenotype, except for the mutation R356W.

(Received 30 July 2008; returned for revision 11 September 2008; finally revised 23 December 2008; accepted 28 January 2009)

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Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder with impaired cortisol secretion.[1,2] More than 90% of cases of CAH are caused by deficiency in the enzyme 21-hydroxylase.[3]

The 21-hydroxylase deficiency impairs production of aldosterone and cortisol and as a result the secretion of the stimulating hormones renin and ACTH is enhanced. The adrenal glands become hyperplastic and produce excess sex hormone precursors that do not require 21-hydroxylation for their synthesis. Therefore, levels of aldosterone and cortisol are decreased and production of adrenal androgens is increased, leading to sodium loss, Addisonian crisis and virilization.[2,4]

Based on severity of symptoms, patients with 21-hydroxylase deficiency may belong to the classical or the nonclassical form of the disease.[1,2,5] The classical form encompasses the complete 21-hydroxylase deficiency, which leads to the effects described above. Partial 21-hydroxylase deficiency leads to the simple virilizing form of CAH, and is characterized by prenatal virilization in females and pseudoprecocious puberty in males. An additional form is mild 21-hydroxylase deficiency. This so-called nonclassical form is mostly asymptomatic or may be associated with signs of postnatal androgen excess consisting of pseudoprecocious puberty, acne, hirsutism and ovarian dysfunction.[5] This makes it difficult to recognize the disease during the neonatal period and therefore these

patients are mostly diagnosed in a later stadium of childhood.[4]

In the Western world, a patient with CAH is most often diagnosed early in life on the basis of screening programmes. Subsequent determination of serum hormone levels and investigation of gene mutations lead to a lifelong therapy with corticosteroids starting at a very early age. Thus, salt loss and Addisonian crises are prevented and postnatal progression of virilization will not occur. This situation differs from that in developing countries, where early diagnosis (new-born screening) and treatment are not routinely available.

Within a large and heterogeneous group of patients with ambiguous genitalia, followed by one of the authors (S.M.H. Faradz), 15 patients suspected of having CAH were identified.

This group of patients offers a unique opportunity to use modern methods to investigate the correlation between phenotype and genotype in untreated CAH patients.

Patients and methods

Patients

The local Medical Ethics Review Committee approved of this study, and informed consent was obtained from all participants, their parents or guardians. Out of a group of 130 patients with disorders of sexual differentiation (accumulated from 1991 until 2004) from the Dr Kariadi University Hospital in Semarang, Central Java, Indonesia, 15 patients were clinically diagnosed as suffering of CAH, based on the 46,XX karyotype in combination with hypervirilization. The ages of these patients varied from 1 to 33 years (Table 1). There were two pairs of siblings (pt 7–10 and pt 5–11). Twelve of the patients had never been treated; three of them (pt 13, 14 and 15) received glucocorticosteroids during a short period before the study.

Methods

Each patient underwent a physical examination and photographs were taken of the genital area. Blood was obtained for determination of hormone concentrations and isolation of DNA.

Phenotype. Most important characteristics were the length of the clitoris, presentation of a urogenital sinus and labial fusion.[6] The photographs of the genital area were evaluated by an independent paediatric urologist (K.P. Wolffenbuttel).

Serum hormones. Levels of the following hormones were analysed in serum: 17-hydroxyprogesterone (17OHP), progesterone, androstenedione, dehydroepiandrosterone sulphate (DHEAS), testosterone, cortisol and renin. Cortisol, progesterone, androstenedione and DHEAS were analysed using luminescence-based immunoassays on the Immulite 2000 (Diagnostic Products Corporation, Los Angeles, CA, USA). 17OHP was analysed using the ^{3}H-radioimmunoassay as described earlier.[7] Testosterone was determined using the Coat-a-Count radioimmunoassay purchased from Diagnostic Products Corporation.

Renin levels were estimated using an immunoradiometric assay purchased from CIS Bio international (Gif sur Yvette, France). Interassay coefficients of variation of those assays were all below 12%.

CYP21A2 mutation analysis. The *CYP21A2* gene was amplified from DNA using the polymerase chain reaction and subsequently sequenced for detection of mutations. The *CYP21A2* gene of each individual patient was sequenced from position -23 up to 90 bases downstream of the stop codon.

PCR amplification of CYP21A2 gene fragments. *CYP21A2* gene-specific amplification was performed using four sets of primers (Table 2).[8–10] Thirty cycles of PCR were carried out in a final volume of 50 µl containing 5 µl (10 ng/µl) genomic DNA of each individual, PCR-buffer containing 1·5 mM $MgCl_2$ (Applied Biosystems, Foster City, CA, USA), 250 µM dNTPs, 3·125 U AmpliTaq DNA Polymerase (Applied Biosystems), 400 nM forward-primer, 400 nM reverse-primer and 5% DMSO. Amplifications were performed using the GeneAmp PCR System 9700 (Applied Biosystems).

The PCR products were purified using the GFX-96 PCR Purification Kit (Amersham Biosciences, Little Chalfont, UK) or the High Pure PCR Product Purification Kit (Roche Applied Science, Mannheim, Germany).

Sequencing the CYP21A2 PCR products. The primers used for sequencing were described earlier.[8–10] Sequencing was performed using a BigDye Terminator v1·1 Cycle Sequencing Kit (Applied Biosystems) to perform the sequence-reactions, a DyeEx 96 Purification Kit (Qiagen, Venlo, the Netherlands) or the Micro-Bio-Spin purification columns (Biorad, Veenendaal, the Netherlands) to purify the fragments and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) to read the sequences. To analyse the sequencing outcome, the computer program Sequencher 4·2 Ink for Windows (Gene Codes Corporation, Ann Arbor, MI, USA) was used.

Restriction fragment length polymorphism (RFLP) and Deletion detection PCR (DDP). DDP was used to detect whether apparent homozygous mutations were because of large deletions in one of the alleles, using DNA of homozygous patients and their parents. We used the techniques described by Asanuma *et al.*[11] to detect hemizygosity in the 5′UTR and that of L'Allemand *et al.*[12] for exon 3. To detect the CYP21-R356W mutation in the parents' DNA, we used the RFLP method.[13]

Statistics. All calculations on the relationships between various parameters were based on Spearman's rho.

Results

Phenotype

Clinical data of the patients are provided in Table 1. All patients had the female social sex, except patient 11 who changed her female gender to male at the age of 20 years. Phenotype was based on

Table 1. Overview of features of the population (age-dependent reference ranges for hormone levels are shown under each age group in italics)

					Phenotype			Serum hormones						
Subject-number	Age (years)		Gender	Karyotype	Clitoris length (cm)	Urogenital sinus	Labia fusion (f)/ partial fusion (pf)	Cortisol (nmol/l)	17OHP (nmol/l)	Prog (nmol/l)	Adion (nmol/l)	DHEAS (μmol/l)	T (nmol/l)	Renin (μU/ml)
1	<7 years	0·2	Female	46,XX	2·5	Yes	pf	302	1169·7	87·2	135	<0·41	7·4	6855·7
2		1	Female	46,XX	3	Yes	pf	117	524·4	29·6	42·2	<0·41	4·4	1838·4
3		2	Female	46,XX	2·5	No	pf	119	273·3	14	>35	<0·41	4·2	141·4
4		4	Female	46,XX	4·1	No	pf	129	428·0	52·2	23·3	<0·41	2·7	224·6
5		3	Female	46,XX	2·8	No	pf	143	664·2	105	15·6	<0·41	2·4	261·4
							N	*200–800*	*<5·0*	*<2·0*	*<2·6*	*<1·4*	*<0·3*	*<60*
6	7–12 years	7	Female	46,XX	5·8	Yes	pf	67·4	551·4	107	138	8·84	16·6	152·5
7		7	Female	46,XX	5	No	pf	172	392·4	35·4	56·6	5·08	6·7	49·5
8		10	Female	46,XX	4·5	No	pf	147	548·1	70·8	130	8·05	18·4	1148·4
9		11	Female	46,XX	3·2	No	pf	310	137·3	34·4	13·6	<0·41	2·2	910·8
10		11	Female	46,XX	3·6	No	no	213	357·8	42·4	74·8	11·4	12·9	172·7
							N	*200–800*	*<5·0*	*<2·0*	*<3·8*	*<5·0*	*<0·6*	*<60*
11	>12 years	17	Male	46,XX	7	Yes	f	205	771·4	192	245·0	16·0	35·7	190·3
12		33	Female	46,XX	3·6	No	pf	122	140·2	21·3	67·4	11·4	11·4	30·5
							N	*200–800*	*<2·0*	*<3·0*	*2–15*	*1–10*	*1–3*	*<60*
									Luteal 4–10	*Luteal 15–70*				
13	Treated	6	Female	46,XX	4·5	No	pf	372	12·7	3·34	4·68	<0·41	0·8	57
14	Treated	7	Female	46,XX	*7*	No	pf	153	0·2	<0·64	<1·05	<0·41	<0·1	20·8
15	Treated	21	Female	46,XX	normal	No	no	89·6	1·0	7·52	1·41	<0·41	0·3	40·6

Table 2. PCR-primers and conditions used in primary amplification

Primerset	Annealing temperature	PCR primer	Position (bp)*	Sequence†
A	69°C	1	-57 to -31	5′-ATGGCTGGGGCTCTTGAGCTATAAGT-3′
		2	1380 to 1405	5′-CCTCAGCTGC**A**TCTCC**A**CG**A**TGTG**A**-3′
B	68°C	3	-265 to -245	5′-AGCTGACTCTGGATGCAGGA-3′
		4	706 to 727	5′-AGCAG**G**GA**GTAGTCTC**CCAA**G**-3′
C	65°C	5	695 to 719	5′-CCGGACCTGTC**C**TTGG**GAGACTAC**-3′
		6	2630 to 2656	5′-GAAAGGCTGCATCTTGAGGATGACAC-3′
D	61°C	7	700 to 720	5′-CCTGTC**C**TTGG**GAGACTAC**T-3′
		8	2892 to 2912	5′-TCTCGCACCCCAGTATGACT-3′

*Reference NC_000006, A from ATG startsite is 1.
†Specific nucleotides for the *CYP21A2* gene are shown by **bold** letters and are underlined.

genital characteristics. Most of the patients had no urogenital sinus and the labia were not or only partially fused. However, most patients had a large clitoris. We decided the clitoris length to be the decisive marker of the severity of virilization. As is indicated in Table 1, only one patient (pt 11) had completely fused labia.

Hormone levels

Hormone levels are also summarized in Table 1. In all patients, levels of progesterone and 17OHP were elevated with the exception of patients 13, 14 and 15, who were treated irregularly with glucocorticosteroids. Cortisol levels were low to low-normal in all patients. Similarly, in all patients (except in the treated patients), levels of progesterone, androstenedione and testosterone were elevated.

In 10 out of the 15 patients, renin levels were elevated, in some of them to more than 100-fold the upper level of normal. Only in two untreated patients (pt 7 and 12), renin levels were within the normal range. As expected, in the three corticosteroid-treated patients, renin levels were normal.

The levels of androstenedione and testosterone correlated significantly with the extent of virilization and clitoris length (see Fig. 1 for the correlation between testosterone and phallus length in nontreated patients), with one exception: patient 10 had high levels of androstenedione and testosterone but suffered from only slightly virilized features. Her younger sister (pt 7) was much more virilized with less elevated levels of androgens. Remarkably, when partial correlations were calculated for the relationships between age, clitoris length and serum testosterone level, only the correlation between testosterone and clitoris length was significant.

It is well known that the conversion of 17-hydroxypregnenolone to dehydroepiandrosterone and 17OHP to androstenedione by the enzyme 17,20-lyase is facilitated by cytochromeB5 (CyB5).[14] Between the age of 1 and 7 years, CyB5 is hardly expressed in the adrenal glands[14] and therefore levels of DHEAS are low. Levels of this adrenal steroid increased after the age of 7 years, as did the

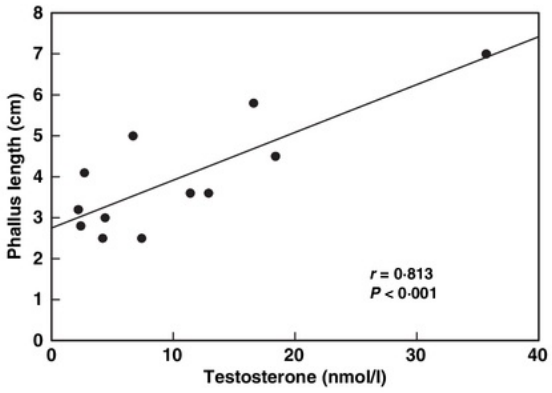

Fig. 1 Relationship between serum testosterone levels and clitoris length in 12 nontreated patients with mutations of *CYP21A2*.

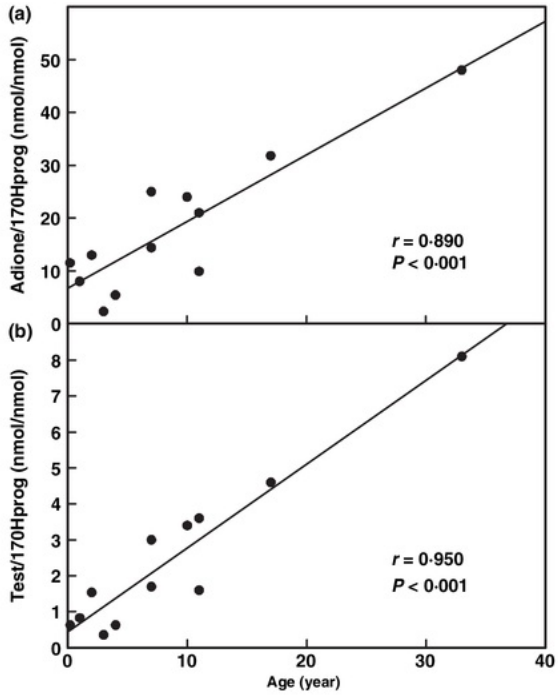

Fig. 2 Age dependence of the ratio between serum androstenedione/17OHP concentrations (a) and serum testosterone/17OHP concentrations (b) in 21-hydroxylase deficient patients (n = 12).

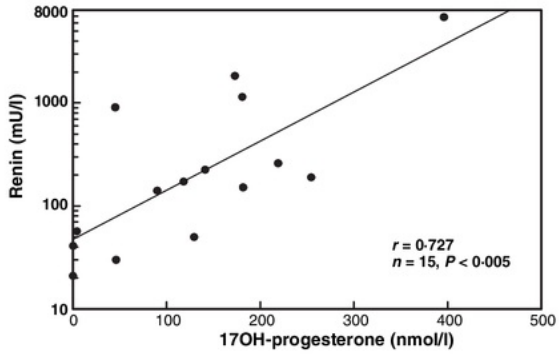

Fig. 3 Relationship between serum levels of renin and 17OHP in 21-hydroxylase-deficient patients.

ratios androstenedione – 17OHP (Fig. 2a), and testosterone – 17OHP (Fig. 2b). These ratios were significantly correlated with age.

Levels of renin correlated with increased levels of 17OHP as explained by the severity of the 21-hydroxylase enzyme deficiency (Fig. 3).

CYP21A2-gene mutation analysis

Table 3 summarizes the mutations in the *CYP21A2* gene detected in each patient. Only mutations that are expected to affect the 21-hydroxylase activity are shown.

Missense and nonsense mutations in exons. In 13 patients, a total of four missense mutations were discovered in exons, which resulted in an amino acid change. These mutations are known to affect the activity of the 21-hydroxylase enzyme and have been described in the literature: P30L, I172N, V281L and R356W (see Table 3). No novel missense mutations were found. In addition, the nonsense mutation Q318X was detected. Patients 2 and 6 were hemizygous for the R356W mutation; both inherited the deleted gene from their mothers and the mutated gene from their fathers. Patients 7

Table 3. *CYP21A2* mutations found in 15 Indonesian patients

Patient	CYP21-mutation	Known mutations which cause a phenotype if homozygous	References	Cortisol/ 17OHP*
7	I172N/I172N	Simple virilizing	20–22	0·438
10	I172N/I172N	Simple virilizing	20–22	0·595
5	IVS2 - 13A > G/IVS2 - 13A > G	Salt-wasting/simple virilizing	17,18	0·215
11	IVS2 - 13A > G/IVS2 - 13A > G	Salt-wasting/simple virilizing	17,18	0·266
1	R356W/R356W	Salt-wasting	1,13,16,24	0·258
2	R356W/-	Salt-wasting	1,13,16,24	0·223
3	R356W/R356W	Salt-wasting	1,13,16,24	0·435
6	R356W/-	Salt-wasting	1,13,16,24	0·122
8	R356W/R356W	Salt-wasting	1,13,16,24	0·268
9	R356W/R356W	Salt-wasting	1,13,16,24	2·258
15	I172N	Simple virilizing	20–22	Received therapy
	R356W	Salt-wasting	1,13,16,24	
12	I172N	Simple virilizing	20–22	0·870
	R356W	Salt-wasting	1,13,16,24	
4	-126C > T -113G > A -110T > C -103A > G -4C > T		12,29,30	
	P30L	Salt-wasting/simple virilizing	12, 29, 30	0·301
	Q318X	Salt-wasting	16,25,26	
13	Q318X IVS2-37A > G/IVS2-37A > G	Salt-wasting	16,25,26	Received therapy
14	V281L F306 + 1nt	Salt-wasting	11	Received therapy
	Q318X IVS2 + 33A > C IVS2 + 67C > T	Salt-wasting	16, 25, 26	

*Reference value >40, see also Table 1 for age-dependent changes.

and 10, who showed homozygosity for the I172N mutation, showed additional heterozygous single nucleotide polymorphisms close to the site of the mutation and were therefore assumed to be homozygous for this mutation.

Intron and 3′UTR mutations. In all patients, a total of 18 intron substitutions were found: 12 substitutions in intron 2, 1 substitution in intron 3, 3 substitutions in intron 6 and 2 substitutions in the 3′UTR. One of these (IVS2 - 13A > G) has been described in the literature as having an effect on enzyme activity. Both affected patients had two mutated alleles. The substitution IVS2 - 37A > G, which has not been described previously, contains the branch site in intron 2 and is likely to affect the formation of the lariat, which is involved in the RNA-splicing mechanism.

5′UTR mutations because of gene-conversion. In patient 4, we found 4 substitutions in the 5′UTR region as a result of a small gene-conversion between the *CYP21A2* gene and the CYP21A1 pseudogene. In addition, a polymorphism was found at position-4 in this patient.

Discussion

In view of the age of the patients at the time of diagnosis, we assumed that the salt-wasting type of CAH would not occur in our study population, as this is considered a life-threatening situation. For this reason, we were surprised to find only two patients with renin levels within the reference range. These two patients had mutations which have been described as causing the simple virilizing form of CYP21 deficiency. Some of the other patients may have compensated the decreased activity of the enzyme by a marked elevation of renin levels. Furthermore, the patients with homo- or hemizygous R356W mutations may have been able to compensate salt-wasting by dietary means or may carry salt-saving mutations in other genes.

We could not determine the degree of virilization at birth because the patients did not visit a medical doctor shortly after birth, except for patient 1. This means that all patients have been exposed to high levels of androgens postnatally resulting in progressive virilization. Fusion of the labia and the presence of a urogenital sinus are prenatally determined and postnatal exposure to androgens will have no further effect. Remarkably all patients received the female gender at birth, suggesting that the patients had only slightly virilized genitals at that time.

The 17OHP levels were more elevated than progesterone in all untreated patients because of conversion of progesterone into 17OHP. The ratios androstenedione/17OHP and testosterone/17OHP were markedly age correlated, probably because of the age-dependent expression of CyB5.[14]

We could not predict the severity of the 21-hydroxylase deficiency based on the renin values, as the values differed in equal genotypes even in siblings. This could be because of 'mature onset', which means that the adrenal glands fail during ageing. Alternatively, this could be explained by variations in genotype/phenotype relationships. However, as expected, the renin levels rise with the increasing levels of 17OHP (Fig. 3).

Most frequent CYP21A2 gene mutations in the Indonesian patients

We have analysed the *CYP21A2* gene of 15 Indonesian CAH patients. To our knowledge, there have been only few reports on *CYP21A2* gene mutations in Asian patients. In 65 CAH families in Taiwan[15] and 28 families in Singapore[16], the most common mutation (40%) was the intron 2 mutation (IVS2 - 13A/C > G). I172N mutations occurred in approximately22% and the R356W mutation in approximately16%. In a study of six Japanese patients, IVS2 - 13A/C > G and I172N were responsible for 58% of the cases of 21-hydroxylase deficiency.[8] Loke *et al.*[16] suggested that the R356W mutation occurs more commonly in the Asian patients both in Singapore and in Taiwan (15–17%) and less in Caucasians (3–9%). The frequency of these mutations described in Caucasian patients is 11–30% for IVS2 - 13A/C > G, 1–30% for I172N and 2–10% for R365W.[3] The mutation IVS2 - 13A > G in the splicing donor site is reported to be the most prevalent mutation appearing in the *CYP21A2* gene among all ethnic groups.[2] In our Indonesian patients, there was a relatively high allele frequency of R356W (40%), I172N (20%) and IVS2 - 13A > G (13%).

CYP21A2 gene mutation analysis; correlation with phenotype

Two patients (a sibling pair) are homozygous for the intron 2 mutation IVS2 - 13A > G (pt 5 and 11). This mutation activates a cryptic splice site that results in aberrant splicing of the pre-mRNA with retention of 19 nucleotides normally spliced out of the pre-mRNA. As a result, a shift in the reading frame occurs which generates a frameshift in the third exon, resulting in a stop codon and the production of a truncated protein.[17,18] In cultured cells, a small amount of normally spliced mRNA is detected. Therefore, a small amount of normal enzyme might be synthesized. As this mutation is associated with the simple virilizing/salt-wasting form, we can explain the phenotype of the two patients.[19]

Four patients had the mutation I172N, of whom two were homozygous (pt 7 and 10, a sibling pair) and two were heterozygous (pt 12 and 15). The I172N mutation is associated with the simple virilizing form of CAH.[20,21] In a study of Tusie-Luna *et al.*,[22] the mutation I172N showed 2% of wild-type activity for 17OHP and progesterone substrates. Km and Vmax values for both 17OHP and progesterone were determined in cellular lysates. When activities were expressed as first-order rate constants Vmax/Km, the activity of the I172N mutant was decreased 200-fold (0·5% activity). Therefore, the phenotype of the sibling pair, patients 7 and 10, can be fully explained. Nevertheless, patient 7 was more virilized than her sister. This difference might be caused by a difference in androgen sensitivity in the two girls; additional variations in the androgen receptor, its coactivators or corepressors could play a role.[23]

Patients 12 and 15 have an additional heterozygous R356W mutation. Chiou *et al.*[24] described a patient who was compound heterozygous for I172N/R356W, having the simple virilizing form of CAH. With the techniques we used, we were not able to

determine whether the heterozygous mutations are located on the same allele. However, if the two mutations would have been present on the same allele, it is likely that the other, intact allele would yield sufficient enzyme to prevent the abnormalities detected in these patients.

Four patients were homozygous for the mutation R356W (pt 1, 3, 8 and 9) and two patients were hemizygous for this mutation (pt 2 and 6). This mutation has a dramatic effect on enzyme functioning,[1,24] associated with the salt-wasting form. In 1990, it was assumed that the protein region involving R356W, constitutes a steroid-binding site.[24] It was speculated that the replacement of Arg356 by Trp might be drastic enough to unfold the protein, which might destroy the steroid-binding capacity of the enzyme leading to a 50-fold lower activity of the enzyme.

Recently, it was discovered that in P450c21, Arg356 is probably located in a region that is involved in redox partner interaction.[2] Stickelbroeck[3] as well as Loke *et al.*[16] found that all CAH patients carrying the homozygous mutation R356W were salt wasters. However, all our six patients have survived 1–11 years in spite of the mutation. In all patients, the Prader stage was III–IV. Furthermore, patients 1, 2 and 6 had a urogenital sinus. If we take the renin values into account, the four highest levels of renin (indicating a severe deficiency of 21-hydroxylase) are detected in patients with the homozygous R356W mutation. We can conclude that the mutation R356W in our patients caused a severe 21-hydroxylase deficiency resulting in the severe virilizing form of CAH.

Patients 4, 13 and 14 are heterozygous for the mutation Q318X. This mutation is predicted to result in a completely nonfunctional enzyme because of premature termination of translation of the mRNA before the conserved heme-binding region of the P450 polypeptide.[25] When the mutated sequence was transfected into mouse Y1 adrenal cells, the resulting mRNA levels were decreased. In the studies of Kharrat *et al.*[26] and Loke *et al.*,[16] all patients who were homozygous for the Q318X mutation had the salt-wasting form. However, Kharrat *et al.* also studied two patients with the simple virilizing form who were compound heterozygous for this mutation, having the mutation R356W or I172N on the other allele.

Patient 14 is heterozygous for F306 + 1nt, V281L and Q318X; all three mutations were found heterozygously in the DNA of the father, whereas none was detected in the mother. Wu and Chung[27] demonstrated a fivefold reduced enzyme activity for the mutant V281L and Stickelbroeck[3] associated this homozygous mutation with the nonclassical form of CAH. Besides that, Stickelbroeck[3] and Asanuma *et al.*[11] reported a cluster mutation in exons 7 and 8 of V281L - F306 + 1nt - Q318X: an extra T nucleotide is inserted in the allele which results in a 100% reduced enzyme activity and consequently in a salt-wasting phenotype. However, as this patient is heterozygous for this cluster mutation, two additional intron 2 substitutions in this patient (IVS2 + 33A > C and IVS2 + 67C > T) could influence the enzyme activity. To prove this, these mutations should be investigated in more detail.

Patient 4 had five additional substitutions in the promoter region, 5′UTR and exon 1, which are compatible with the sequence of the pseudogene CYP21A1 and can decrease the transcriptional activity to 20% of that of CYP21A2.[28] It seems likely that a hybrid was formed between CYP21A1 and *CYP21A2*, with a junction site just before intron 2. This hybrid would be formed by unequal crossover, resulting in a deletion of the 5′ end of the *CYP21A2* gene, the *C4B* gene and the 3′ end of the CYP21A1 pseudogene.[11,29] In addition, the mutation P30L causes an enzymatic activity of 60%.[30]

The heterozygous combination of the mutations together with Q318X in patient 4 explains the phenotype.

Besides the heterozygous Q318X mutation, we found in patient 13 the homozygous substitution IVS2 - 37A > G, which contains the branch site in intron 2. This substitution has not been described previously and may affect the formation of the lariat during RNA splicing,[31] which results in intron 2 retention or exon 2 skipping. This may have an important effect on the phenotype.

In conclusion, we found mutations in the *CYP21A2* gene in all patients with a relatively high allele frequency of R356W (40%), I172N (20%) and IVS2 - 13A > G (13%). Most remarkable in this study is the high frequency of the mutation R356W, which is very often described as causing the life-threatening salt-wasting form of congenital adrenal hyperplasia. Apparently patients having the R356W mutation were able to compensate salt wasting by dietary means or may carry salt-saving mutations in other genes. The 21-hydroxylase deficiency resulted in postnatal progressive virilization and almost all patients had the female gender and a strong desire to have a female appearance. Therefore, a project has been initiated to offer medical and surgical treatment.

Acknowledgements

The authors thank the Gender team of the Medical Faculty and the Molecular and Cytogenetic Laboratory of the Diponogoro University/Dr Kariadi Hospital for help in research of the patients and the karyotyping.

Competing interests/financial disclosure

Nothing to declare.

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